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Protein phosphatase 2A inhibition and circumvention of cisplatin cross-resistance by novel TCM-platinum anticancer agents containing demethylcantharidin

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Abstract—Novel TCM–platinum compounds $[Pt(C_8H_8O_5)(NH_2R)_2]$ **1**–**5**, derived from integrating demethylcantharidin, a modified component from a traditional Chinese medicine (TCM) with a platinum moiety, possess anticancer and protein phosphatase 2A inhibition properties. The compounds are able to circumvent cisplatin resistance by apparently targeting the DNA repair mechanism. Novel isosteric analogues $[Pt(C_9H_{10}O_4)(NH_2R)_2]$ **A** and **B**, devoid of PP2A-inhibitory activity, were found to suffer from an enhanced DNA repair and were cross-resistant to cisplatin. The results advocate a well-defined structure–activity requirement associating the PP2A-inhibiting demethylcantharidin with the circumvention of cisplatin cross-resistance demonstrated by TCM–Pt compounds **1**–**5**.

1. Introduction

Cisplatin and carboplatin (Fig. 1) are effective platinum (Pt)-based anticancer agents with a broad spectrum of activity against solid tumors. However, therapeutic responses vary among patients, and the emergence of resistance is frequently encountered.[1,2] Resistance to cisplatin has been studied extensively and found to involve one or more of the following events: decreased accumulation of the drug; increased cellular detoxification by glutathione (GSH) or metallothioneins; increased removal of cisplatin DNA adducts and enhanced DNA repair; tolerance to platinum-DNA damage; and alterations in signal transduction pathways involved in apoptosis activation that occur in tumor cells following drug exposure.[3]

A major goal in designing new Pt-based anticancer agents is to circumvent cisplatin resistance. We recently reported the development of a novel series of platinum anticancer agents $[Pt(C_8H_8O_5)(NH_2R)_2]$ **1**–**5** (Fig. 1), by incorporating into the Pt moiety a modified structural component of a traditional Chinese medicine (TCM), demethylcantharidin (DMC).[4] The TCM–Pt compounds were found to be highly cytotoxic, apparently able to overcome Pt resistance and demonstrated protein phosphatase 2A (PP2A) inhibitory activity.[4]

To our knowledge, inhibition of protein phosphatases is a new property for Pt-based anticancer compounds with potential or proven therapeutic value, and might explain the high potency of the TCM–Pt compounds that has been demonstrated in vitro and in vivo.[4] TCM–Pt compounds **1**–**5** were proposed to have a dual mechanism of cytotoxic action: (i) inhibition of PP2A by the diacid form of demethylcantharidin that is slowly released; and (ii) platination of DNA by the Pt moiety. The

Abbreviations: DMC, demethylcantharidin; DACH, diaminocyclohexane; NER, nucleotide excision repair; PP1 and PP2A, protein phosphatase 1 and 2A, respectively; TCM, traditional Chinese medicine.
Keywords: Demethylcantharidin; Protein phosphatase inhibition; Cisplatin resistance; Nucleotide excision repair.
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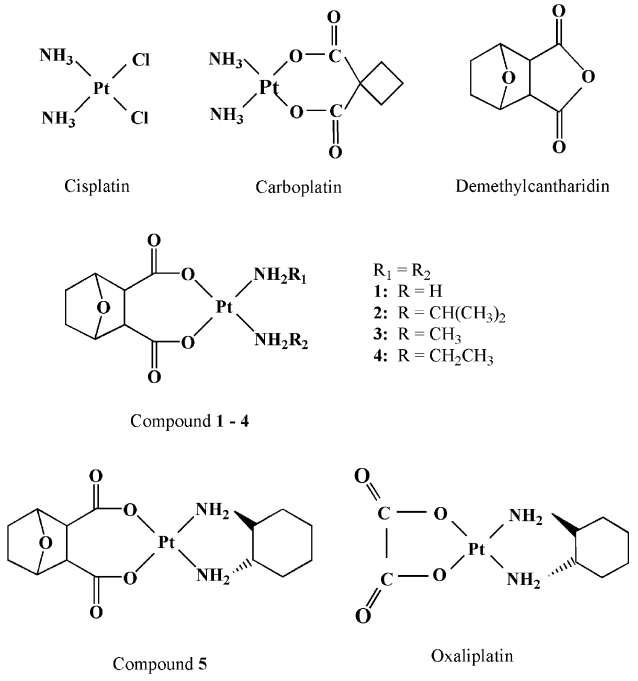


Figure 1. Chemical structures of cisplatin, carboplatin, oxaliplatin, novel TCM–Pt compounds **1**–**5** and demethylcantharidin.

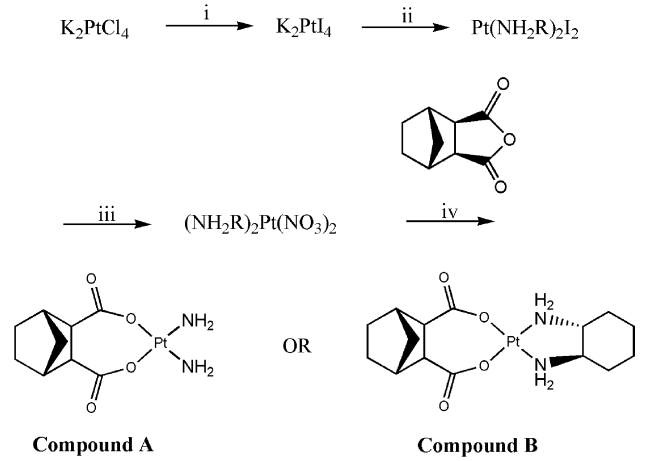
former is also believed to be responsible for the apparent circumvention of Pt resistance.

Recent literature findings suggest that PP2A is essential for the nucleotide excision repair (NER) mechanisms, whereby its inhibition can hijack the major repair mechanism to DNA-Pt lesions.[5] Therefore, it is feasible that TCM–Pt [**1**–**5**] or demethylcantharidin on its own, might preferentially target the nucleotide excision repair mechanism, and if proven, the compounds could be beneficial in treating cisplatin-refractory tumors.

The aim of the present study was to verify the unique PP2A inhibitory activity of the TCM–Pt compounds, and to scrutinize the role of demethylcantharidin in inhibiting the repair of DNA-Pt adducts, likely to be the reason for the circumvention of cisplatin cross-resistance. To illustrate the importance of incorporating the PP2A-inhibiting demethylcantharidin into the TCM–Pt compounds, an isosteric isomer of demethylcantharidin (ligand **C**) and two new platinum compounds (**A** and **B**; which are analogues of compounds **1** and **5**, respectively) were synthesized (Scheme 1) and biologically evaluated.

2. Results and discussion

TCM–Pt compounds **1**–**5** were synthesized by reacting demethylcantharidin (DMC) with a series of $(NH_2R)_2Pt(NO_3)_2$ as described previously.[4] *exo*-Bicyclo[2.2.1]heptane-2,3-dicarboxylic anhydride (Ligand **C**), a structural modification of demethylcantharidin by replacing the bridge oxygen atom in the latter with an isosteric methylene group, was isolated from the isomerization of the *endo*-hept-5-ene adduct, readily



Scheme 1. (i) KI; (ii) NH_3 (**A**), *trans*-(±)-$C_6H_{10}(NH_2)_2$ (**B**); (iii) $AgNO_3$; (iv) NaOH.

prepared from a Diels–Alder reaction between cyclopentadiene and maleic anhydride, followed by hydrogenation.[6] Spectral characterization of all intermediates to Ligand **C** concurred with that reported in literature.[7] New platinum compounds $[Pt(C_9H_{10}O_4)(NH_2R)_2]$ **A** and **B**, respective analogues of TCM–Pt compounds **1** (where R = H), and **5** (where R = *trans*-C_6H_{10}), were prepared by reacting ligand **C** with the appropriate Pt moieties (Scheme 1).

The dissimilar patterns of biological behavior between the classical Pt-based anticancer drugs cisplatin and carboplatin, and TCM–Pt compounds **1**–**5** suggest that they may operate under different mechanisms of anticancer activity and/or resistance.[4] Spearman rank order analysis was performed to compare the sensitivity pattern of the TCM–Pt compounds with that of established Pt-based anticancer drugs (Table 1). The statistical method is similar to the COMPARE algorithm adopted for data analysis in the National Cancer Institute (NCI) human tumor cell line (60-cell) screening.[8,9] Briefly, the novel compounds were ranked for similarity in their in vitro cell growth inhibition pattern in a panel of tumor cell lines, to that of a selected probe or seed compound. Similarity of pattern to that of the seed is expressed quantitatively as a correlation coefficient. Compounds high in the ranking may possess a mechanism of action similar to that of the seed compound.[10] Cisplatin sensitivity was found to correlate only with carboplatin, which is consistent with clinical findings that these two drugs share the same spectrum of antitumor activity.[11] However, cisplatin and the novel TCM–Pt compounds were not correlated, mainly due to the fact that compounds **1**–**5** were also active against cisplatin-unresponsive cancer cells such as hepatocellular carcinoma. Interestingly, rank order of sensitivity to oxaliplatin was found to correlate with that of compound **5**. This implies that the inclusion of the diaminocyclohexane (DACH) ring structure into compound **5** may have introduced a similar mechanism of action to oxaliplatin, which has demonstrated antitumor activity in cisplatin-resistant murine L1210 leukemia cells.[12]

Table 1. Spearman rank order analysis of relative sensitivity of 10 cancer cell lines[a] to demethylcantharidin and different Pt compounds (Cpd)

Compounds	All cell lines Spearman correlation (rho)	Significance (*P* value)
Cisplatin versus carboplatin	0.952	<0.0005[b]
Cisplatin versus cpd **1**	0.564	0.090
Cisplatin versus cpd **2**	0.467	0.174
Cisplatin versus cpd **3**	0.612	0.060
Cisplatin versus cpd **4**	0.600	0.067
Cisplatin versus cpd **5**	0.576	0.082
Cisplatin versus DMC	−0.024	0.947
Carboplatin versus cpd **1**	0.467	0.174
Carboplatin versus cpd **2**	0.430	0.214
Carboplatin versus cpd **3**	0.588	0.074
Carboplatin versus cpd **4**	0.515	0.128
Carboplatin versus cpd **5**	0.418	0.229
Carboplatin versus DMC	−0.091	0.802
Oxaliplatin versus cisplatin	0.462	0.179
Oxaliplatin versus carboplatin	0.430	0.214
Oxaliplatin versus cpd **1**	0.621	0.237
Oxaliplatin versus cpd **2**	0.587	0.198
Oxaliplatin versus cpd **3**	0.654	0.289
Oxaliplatin versus cpd **4**	0.572	0.229
Oxaliplatin versus cpd **5**	0.921	<0.0005[b]
Cpd **1** versus cpd **2**	0.964	<0.0005[b]
Cpd **1** versus cpd **3**	0.903	<0.0005[b]
Cpd **1** versus cpd **4**	0.915	<0.0005[b]
Cpd **1** versus cpd **5**	0.939	<0.0005[b]
Cpd **1** versus DMC	0.462	0.179
Cpd **2** versus cpd **3**	0.879	0.001[b]
Cpd **2** versus cpd **4**	0.830	0.002[b]
Cpd **2** versus cpd **5**	0.855	0.002[b]
Cpd **2** versus DMC	0.413	0.235
Cpd **3** versus cpd **4**	0.952	<0.0005[b]
Cpd **3** versus cpd **5**	0.794	0.002[b]
Cpd **3** versus DMC	0.535	0.111
Cpd **4** versus cpd **5**	0.867	0.001[b]
Cpd **4** versus DMC	0.565	0.089
Cpd **5** versus DMC	0.456	0.185

[a] The cancer cell lines include L1210 mouse leukemia, COLO320DM human colon cancer, SK-Hep-1 human liver cancer, Hep-G2 human liver cancer, MDA-MB-231 human breast cancer, NCI:H460 human nonsmall cell lung cancer, SK-OV-3 human ovarian cancer, NTERA-S cl D1 human testicular cancer, and two primary culture of human gastric cancer.

[b] a *P* value of <0.002 for 28 individual tests is equivalent to $P<0.05$ for a single test by Bonferroni criteria.

In this study, the cisplatin-resistant mouse leukemia cell line (L1210/CDDP) was developed over a time period exceeding 18 months, and at the time of experimentation, L1210/CDDP cells were 30-fold resistant to cisplatin and 12-fold resistant to carboplatin. Inhibition of the divalent cation-independent protein phosphatases (PP1 and PP2A) activity in cell homogenates of sensitive parental L1210/0 and cisplatin-resistant L1210/CDDP by TCM–Pt compounds and DMC were determined by a malachite green colorimetric assay.[13–15] After 24-h preincubation in normal saline, the IC_{50} for the protein phosphatase inhibition ranged from 31.3 μM for compound **5** to 132.3 μM for **2** in L1210/0; and 27.2 μM for **5** to 150.2 μM for **2** in L1210/CDDP cell homogenates (Table 2). For the control DMC, the IC_{50} was 25.1 μM in L1210/0 and 21.2 μM in L1210/CDDP. Compounds **1**–**5** were pre-incubated in normal saline because they did not exhibit any protein phosphatase inhibition when freshly prepared in aqueous solutions;[4] but there is a gradual increase in their protein phosphatase inhibitory effect due to the progressive release of hydrolyzed demethylcantharidin after incubation in normal saline over a period of 24 h.[16] The results are in sharp contrast to cisplatin and carboplatin where it had previously been established to have no protein phosphatase inhibitory activity.[4] However, there was no significant difference in the protein phosphatase inhibitory activity of compounds **1**–**5** between the sensitive and resistant L1210 cells. In a cisplatin-refractory hepatocellular carcinoma cell line SK-Hep-1, compounds **1**–**5** give a similar trend of protein phosphatase inhibition to L1210, but with generally lower IC_{50}s (Table 2). Ligand **C** and novel compounds **A** and **B** were also examined for protein phosphatase inhibitory activity. When freshly prepared or after pre-incubation in normal saline for 24 h, none of the three compounds showed protein phosphatase inhibitory activity at concentrations up to 1 mM. Accordingly, we assert that the bridged oxygen atom in the 7-oxabicycloheptane ring of demethylcantharidin is crucial for PP2A and/or PP1 inhibition and it is consistent with the findings reported by McCluskey et al.[17]

At present, the dominant mechanism accounting for either de novo or clinically acquired resistance is unclear. It follows that if the TCM–Pt compounds could circumvent cisplatin resistance by interfering with some of the resistance mechanisms, then it would certainly be a major advancement in Pt-based anticancer chemotherapy. We therefore examined the possible targeting of the nucleotide excision repair mechanism by compounds **1**–**5**, and attempted to clarify its relationship with PP2A inhibition.

Table 2. Protein phosphatases (PP2A + PP1) inhibitory activity[a] of compounds **1**–**5**[b] and demethylcantharidin (DMC) in sensitive parental L1210/0, SK-Hep-1/0, and resistant L1210/CDDP, and SK-Hep-1/$CDDP_{10}$ cell homogenates (IC_{50}/μM)[c]

IC_{50}	Cisplatin	Carboplatin	**1**	**2**	**3**	**4**	**5**	DMC
L1210/0	NA[d]	NA[d]	60.5	132.3	71.3	82.2	31.3	25.1
L1210/CDDP	NA[d]	NA[d]	63.3	150.2	70.2	90.5	27.2	21.2
SK-Hep-1/0	NA[d]	NA[d]	29.5	78.1	49.3	52.2	22.1	18.2
SK-Hep-1/$CDDP_{10}$	NA[d]	NA[d]	25.3	62.8	50.2	48.5	19.2	16.1

[a] Inhibition of cellular protein phosphatases was evaluated using K-R-pT-I-R-R as the substrate (PP2A assay kit, Upstate Biotechnology).

[b] All compounds were preincubated in normal saline at 37 °C for 24 h.

[c] Results represent mean of three independent experiments.

[d] No activity.

Although inhibition of both PP1 and PP2A was evaluated in our assay, it is still reasonable to conclude that inhibition of PP2A is responsible for the circumvention of cisplatin resistance by compounds **1**–**5**. Demethylcantharidin, like other cantharidin analogues, shows moderate PP2A selectivity (PP2A IC_{50} = 0.37 μM; PP1 IC_{50} = 1.98 μM; PP2A selectivity = 5.5).[18] In fact, the inhibition of PP1 and PP2A by their inhibitors is known to occur competitively at a single site on each enzyme.[19] Most of the inhibitors do not demonstrate differential selectivity towards the two enzymes due to the considerable sequence homology between PP1 and PP2A.[20] The main exception to this generalization is the ~100-fold and ~40,000-fold selective inhibition of PP2A by okadaic acid and fostriecin, respectively.[18,21] On the other hand, highly selective inhibitors of PP1 have proven to be rather elusive, with tautomycin[22] and the modified microcystin analogues[23] (which are only 5–10-fold selective for PP1 over PP2A) being the only available small molecule PP1 inhibitors. Recently, McCluskey et al. have reported two novel cantharidin analogues exhibiting PP1 selectivity of 30–40-fold.[24] However, the increase in selectivity did not arise from an increased potency at PP1, but at the expense of inhibition of PP2A. Therefore, only the PP2A highly specific okadaic acid was employed in our study to verify the specific role of PP2A in the circumvention of cisplatin resistance, the result of which will be illustrated below. Importantly, PP2A has been shown to be necessary for nucleotide excision repair in vitro.[5] Repair was more sensitive to okadaic acid than to tautomycin, suggesting the involvement of a PP2A-type enzyme; and was insensitive to inhibitor-2, which exclusively inhibits PP1-type enzymes. Consistent with this, highly purified PP2Ac was able to restore repair capacity to okadaic acidic- and microcystin-LR-inhibited cell extracts, while the addition of PP1γ did not have any effect.

By utilizing a resistant L1210 cell line (L1210/CDDP) where mechanisms of cisplatin-resistance have been well characterized,[25] the antiproliferative activity of the platinum compounds, DMC and ligand **C** was determined using a colorimetric MTT assay.[26] TCM–Pt [**2**–**5**] demonstrated no or negligible cisplatin cross-resistance in L1210/CDDP cells (Table 3). However, compound **1** did exhibit a low level of cisplatin cross-resistance, about 3-fold in L1210/CDDP; this is not unexpected as the structure is similar to cisplatin, and upon cleavage of the DMC ligand, the remaining Pt moiety is essentially the same as that formed from cisplatin (Fig. 1). The low degree of resistance exhibited by compound **1** appears to be due to a reduced cellular accumulation of Pt (data not shown). The structure of compound **5**, previously shown to be the most potent in in vitro antiproliferative screening, consists of a diaminocyclohexane (DACH) carrier ligand and the demethylcantharidin leaving group, which were deliberately designed in to combat cisplatin resistance. The DACH ligand, as in oxaliplatin, has been demonstrated to hinder DNA repair by preventing or reducing the binding of specific damage repair proteins such as the mismatch repair enzyme complex, thereby decreasing the replicative bypass of platinum-DNA adducts.[27] Indeed, compound **5** did

Table 3. Antiproliferative activity[a] of compounds **1**–**5** towards sensitive parental and cisplatin-resistant L1210 mouse leukemia and SK-Hep-1 human hepatocellular carcinoma (resistance level for the resistant subline is shown in parenthesis)[b]

IC_{50} (μM)	Cisplatin	Carboplatin	DMC	Cisplatin + 6 μM DMC[c]	Ligand **C**	**1**	**A** (analogue of **1**; with integration of ligand **C**)	**2**	**3**	**4**	**5**	**B** (analogue of **5**; with integration of ligand **C**)
L1210												
Sensitive	1.01 ± 0.04	17.78 ± 2.05	13.73 ± 1.62	1.03 ± 0.20	386.5 ± 21.2	5.97 ± 0.44	5.90 ± 0.76	11.54 ± 2.71	12.66 ± 2.79	12.24 ± 1.85	0.12 ± 0.05	1.07 ± 0.06
Resistant	31.03 ± 2.32	217.4 ± 13.74	12.56 ± 2.10	6.37 ± 1.25	391.6 ± 26.4	16.01 ± 1.05	168.1 ± 15.2	12.96 ± 2.76	12.57 ± 0.54	13.05 ± 1.22	0.12 ± 0.04	2.33 ± 0.14
	(30.7)	(12.2)	(0.9)	(6.2)	(1.0)	(2.7)	(28.5)	(1.1)	(1.0)	(1.1)	(1.0)	(2.2)
SKHep1												
Sensitive	47.73 ± 3.96	472.3 ± 39.26	11.48 ± 4.47	45.18 ± 3.75	505.2 ± 30.2	11.09 ± 2.31	81.23 ± 6.95	13.88 ± 2.65	17.23 ± 3.56	16.84 ± 4.23	3.99 ± 0.52	25.16 ± 2.03
Resistant	249.63 ± 20.35	1653.05 ± 44.23	11.02 ± 3.21	48.23 ± 5.16	492.1 ± 31.6	11.73 ± 1.62	349.2 ± 25.3	13.55 ± 3.22	18.16 ± 4.23	19.48 ± 2.56	3.92 ± 0.42	37.74 ± 2.95
	(5.2)	(3.5)	(1.0)	(1.1)	(1.0)	(1.1)	(4.3)	(1.0)	(1.1)	(1.2)	(1.0)	(1.5)

[a] IC_{50} is the drug concentration effective in inhibiting 50% of the cell growth measured by MTT assay after 72-h drug exposure (IC_{50}/μM ± sd; n = 12–16).
[b] Resistance level (n-fold) in parenthesis. The fold resistance equals the IC_{50} of the resistant cells divided by the IC_{50} of parental cells for individual drugs.
[c] 6 μM of demethylcantharidin alone does not have any antiproliferative activity on the cells.

not exhibit any cross-resistance to cisplatin, and combined with its superior potency, exemplifies the potentially powerful combination of the diaminocyclohexane and demethylcantharidin structural entities.

Demethylcantharidin alone or in combination with cisplatin was similarly investigated. In L1210, demethylcantharidin did not show cross-resistance with cisplatin. But when cells were treated with a combination of demethylcantharidin (6 μM) and cisplatin, the IC_{50}s were 1.03 μM in L1210/0 and 6.37 μM in L1210/CDDP, changed from 1.01 and 31.03 μM, respectively, from treatment with cisplatin alone (Table 3). Treatment of L1210 cell lines with 6 μM of demethylcantharidin alone was found to be nontoxic (data not shown). The dose modification factor (DMF), estimated by the ratio of the IC_{50} without demethylcantharidin divided by that with demethylcantharidin, was 0.98 for L1210/0 and 4.87 for L1210/CDDP cells. To ascertain the specificity of PP2A inhibition (but not PP1) in the circumvention of cisplatin resistance, the highly PP2A-specific (100-fold over PP1) okadaic acid was also evaluated in combination with cisplatin. Concomitant treatment of the resistant cells with cisplatin plus okadaic acid (at a concentration as low as 10 nM) significantly reduced the IC_{50} to a level similar to that in the sensitive cells, from treatment with cisplatin alone (data not shown). Therefore, it is highly likely that the inhibition of PP2A, but not PP1, with overlapping substrate specificity is responsible for the circumvention of cisplatin resistance.

TCM–Pt [**1**–**5**] were also tested against a human hepatocellular carcinoma SK-Hep-1 cell line (SK-Hep-1/$CDDP_{10}$) where cisplatin resistance has been acquired through intermittent drug exposure. Significantly, compounds **1**–**5** and DMC were all not cross-resistant to cisplatin (Table 3). Antiproliferative activity of compounds **A** and **B** was also evaluated. Compounds **A** and **B** were found to be less potent in L1210/CDDP than in L1210/0 cells (demonstrating about 28.5-fold and 2.2-fold resistance, respectively). Moreover and significantly, concomitant treatment of the cells with cisplatin plus ligand **C** (up to 100 μM) did not reduce the IC_{50} to that treated with cisplatin alone.

The significant cross-resistance of **A** with cisplatin (28-fold in L1210/CDDP and 4-fold in SK-Hep-1/$CDDP_{10}$) demonstrated the importance of incorporating the PP2A-inhibitory DMC moiety into compounds **1**–**5**. Whereas the lower cross-resistance of **B** (about 2-fold in L1210/CDDP and 1.5-fold in SK-Hep-1/$CDDP_{10}$) with cisplatin does reinforce further, the powerful combination of DMC with the DACH moiety (as in compound **5**) in overcoming cisplatin cross-resistance.

Reduced total cellular Pt accumulation and increased cytoplasmic detoxification by glutathione were previously established to be operative in our cisplatin-resistant L1210 model.[4] In this current study, our aim was to examine the possible targeting of the DNA repair capacity as the primary mechanism of cisplatin resistance by compounds **1**–**5**. For Pt-based anticancer drugs, there are literature reports that support the nucleotide excision repair (NER) mechanism as being the more important factor in causing cisplatin resistance in vitro.[28,29]

L1210/0 and L1210/CDDP cells were treated with platinum compounds (100 μM) followed by washing with PBS. The cells were then frozen at −80 °C (zero time control) or re-incubated in drug-free medium for different time courses to allow for repair. DNA was then isolated using DNAzol® reagent. The total DNA bound platinum was estimated by atomic absorption spectroscopy, and repair was calculated by comparing the platinum content for the repair samples with that in the control. Measuring the removal of platinum adducts from DNA serves as a useful indicator of the NER mechanism in action.[5]

In the DNA repair assays, different rates of removal of DNA-Pt adducts were observed: 18% and 48% were removed within the first 2 h of recovery in L1210/0 and L1210/CDDP cells that were treated with cisplatin (Fig. 2A). However, for compounds **2** and **5**, there was no significant difference in the rate of removal of the DNA-Pt adducts from the resistant and the sensitive cells (Fig. 2B and C): less than 10% of DNA-Pt adducts was removed within the first 2 h of recovery in L1210/0 and L1210/CDDP cells. For compounds **A** and **B**, less DNA platinated adducts were formed in the cisplatin-resistant than the sensitive parental L1210 cells (**A**, 7.8 vs 18.2; **B**, 9.2 vs 19.8 pmol Pt/μg DNA). As found with cisplatin, a significantly faster rate of removal of DNA-Pt adducts was observed in the L1210/CDDP than the L1210/0 cells for compounds **A** and **B**, where approximately 20% and 17% of DNA-Pt adducts were removed from L1210/0, and about 49% and 48% were removed from L1210/CDDP for **A** and **B**, respectively, within the first 2 h of recovery (Fig. 2D and E).

In this study, enhanced DNA repair in the resistant L1210 cell line occurred only with cisplatin, and compounds **A** and **B**, which had no PP2A inhibitory effect. DNA repair in cells treated with the PP2A-inhibiting TCM–Pt compounds **2** and **5** was much less effective. More significantly, the role of demethylcantharidin in compounds **1**–**5** was separately confirmed by the in situ addition of demethylcantharidin into resistant cells. Retardation of DNA repair was observed in L1210/CDDP cells exposed to cisplatin in combination with demethylcantharidin, where about 17% cisplatin-DNA adducts were removed, compared to 48% with treatment with cisplatin alone (Fig. 3A). However, a similar retardation of DNA repair was not observed in the L1210/0 cells (Fig. 3B). The combination of cisplatin and demethylcantharidin resulted in increased cytotoxicity, and appeared to have usurped the enhanced repair in the resistant cells by inhibiting the removal of DNA-Pt adduct, thus enabling circumvention of resistance. Parallel experiments in L1210/CDDP cells using a combination of cisplatin and the nonPP2A inhibiting ligand **C**, or 1,1-cyclobutanedicarboxylate, the leaving group in carboplatin, showed an absence of this circumvention of resistance (data not shown).

Therefore, the results strongly advocate the role of demethylcantharidin to be that of a protein phosphatase

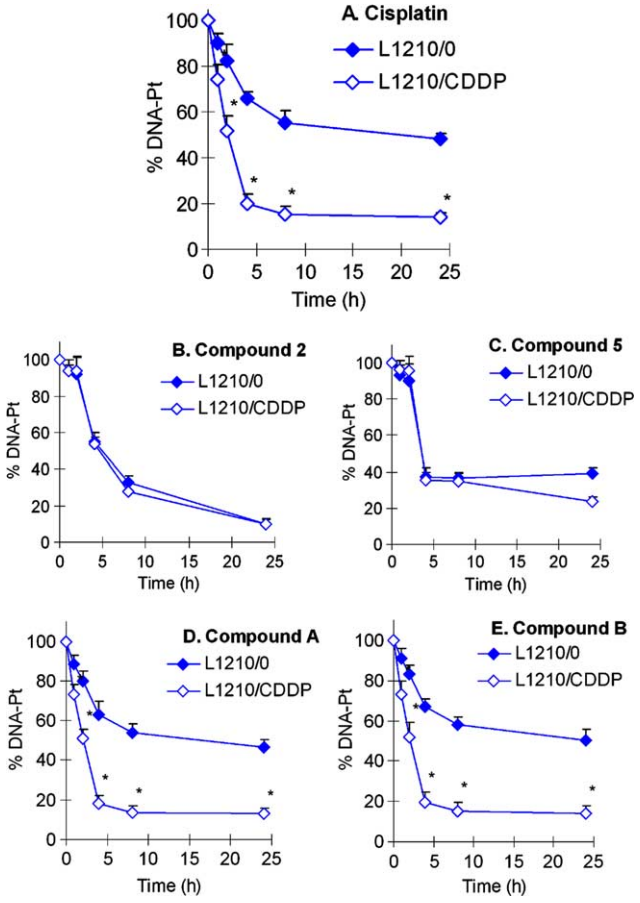


Figure 2. Repair of DNA-Pt adduct measured as the loss of Pt from cellular DNA with time when cells previously exposed to cisplatin (A), compound **2** (B), compound **5** (C), compound **A** (D), or compound **B** (E) for 4 h were incubated in drug-free medium. The DNA-Pt content of the cells (pmol Pt/μg DNA) at the time just after the 4 h drug treatment was as follows: after cisplatin: L1210/0 44.1 ± 5.6, L1210/CDDP 19.9 ± 2.2; after compound **2**: L1210/0 7.9 ± 1.5, L1210/CDDP 8.1 ± 1.1; after compound **5**: L1210/0 21.0 ± 3.6, L1210/CDDP 21.3 ± 3.6; after compound **A**: L1210/0 18.2 ± 3.1, L1210/CDDP 7.8 ± 1.6; and after compound **B**: L1210/0 19.8 ± 2.3, L1210/CDDP 9.2 ± 1.9 ($n = 3$). ($^{*}p < 0.05$; difference between L1210/CDDP and L1210/0).

2A inhibitor in overcoming cisplatin resistance, and in preferentially attacking the nucleotide excision repair mechanism. This result is consistent with our previous finding that demethylcantharidin or more accurately, its hydrolyzed form, is progressively released from the TCM–Pt compounds in aqueous solutions.[16] The released demethylcantharidin is believed to be responsible for the circumvention of cisplatin resistance.

3. Conclusion

In summary, the structure of demethylcantharidin with the oxygen bridge intact, is essential for the lack of cisplatin resistance demonstrated by TCM–Pt compounds **1**–**5**. Retention of antitumor activity against cisplatin-resistant cell lines suggests the compounds are mechanistically different from the classical cisplatin analogues. The results strongly suggest the TCM–Pt compounds and demethylcantharidin do target selectively the

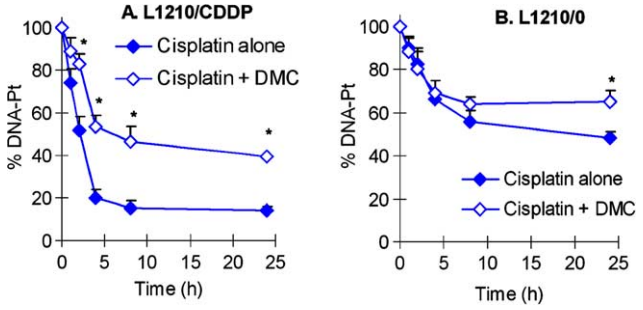


Figure 3. Repair of DNA-Pt adduct measured as the loss of Pt from cellular DNA with time when cells previously exposed to cisplatin alone or combination of cisplatin and demethylcantharidin (6 μM) in L1210/CDDP (A) or L1210/0 (B) for 4 h were incubated in drug-free medium. The DNA-Pt content of the cells (pmol Pt/μg DNA) at the time just after the 4 h drug treatment was as follows: after cisplatin alone: L1210/0 44.1 ± 5.6, L1210/CDDP 19.9 ± 2.2; after combination of cisplatin and demethylcantharidin (6 μM): L1210/0 51.6 ± 4.5, L1210/CDDP 33.0 ± 4.4. ($^{*}p < 0.05$: Difference between treatment with cisplatin alone and combination of cisplatin and DMC).

nucleotide excision repair mechanism, with inhibition of PP2A playing a major role. Since it has been implicated that DNA repair may be the most important factor in cisplatin resistance, the TCM–Pt compounds and/or demethylcantharidin might prove clinically useful in circumventing resistance in cisplatin-refractory tumors. We are currently evaluating the effect of the novel TCM–Pt compounds and demethylcantharidin on the cellular expression of ERCC1 and XPA proteins that actively participate in the NER mechanism. We are also conducting an in-depth investigation into the potential sensitization of cisplatin-resistant cells to treatment with cisplatin by demethylcantharidin.

4. Experimental

Cisplatin and carboplatin were obtained from Strem (Newburyport, USA). 1,1-Cyclobutanedicarboxylic acid was from Aldrich Chemical Company (St Louis, MO, USA). DNAzol® DNA extraction reagent was obtained from Life Technologies (Grand Island, NY, USA). Protein phosphatase PP2A assay kit was purchased from Upstate Biotechnology Incorporation (Lake Placid, N.Y., USA). All other chemicals and reagents were of the best grade available.

Melting or decomposition points were determined on a Griffin melting point apparatus and were not corrected. Spectra were obtained as follows: IR spectra were recorded on a Perkin–Elmer Spectrum BX FT-IR spectrometer; ^{1}H and ^{13}C NMR spectra (500 MHz) were determined on a Bruker ARX-500 high resolution spectrometer; high resolution mass spectra were recorded on a Bruker TOF mass spectrometer. Elemental analyses (C, H, N) were performed at the Shanghai Institute of Organic Chemistry, PR China.

4.1. Synthesis of TCM–Pt compounds

Demethylcantharidin was readily prepared from a Diels–Alder reaction between furan and maleic

anhydride; and TCM–Pt compounds **1**–**5** were synthesized by reacting demethylcantharidin with a series of $(NH_2R)_2Pt(NO_3)_2$ as described previously.[4]

4.1.1. *endo*-Bicyclo[2.2.1]hept-5-ene-2,3-dicarboxylic anhydride. Maleic anhydride (80 g, 0.816 mol) was dissolved in ethyl acetate (250 mL), followed by addition of petroleum ether (65–95 °C, 100 mL) and the mixture cooled in an ice bath. Freshly distilled cyclopentadiene (59 g, 0.9 mol) was added with stirring and a white solid appeared. The reaction mixture was further stirred at room temperature for 4 h, filtered and the intermediate *endo*-bicyclo[2.2.1]hept-5-ene-2,3-dicarboxylic anhydride recrystallized from 1:1 ethyl acetate/petroleum ether (85.23 g, 63%); mp 164–166 °C; IR (KBr) ν_{max} 1859, 1790, 1231, 1089, 905 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.78 (2H, m, 7-H_2), 3.51 (2H, m, 1-H and 4-H), 3.58 (2H, m, 2-H and 3-H), 6.31 (2H, m, 5-H and 6-H) ppm; ^{13}C NMR ($CDCl_3$) δ 40.70 (C-7), 47.66 (C-1 and C-4), 53.34 (C-2 and C-3), 136.13 (C-5 and C-6), 171.88 (CO) ppm.

4.1.2. *exo*-Bicyclo[2.2.1]hept-5-ene-2,3-dicarboxylic anhydride. *endo*-Bicyclo[2.2.1]hept-5-ene-2,3-dicarboxylic anhydride was isomerized to the *exo*-adduct according to the method of Craig.[6] In brief, the *endo*-adduct (50 g, 0.305 mol) was heated in an open flask immersed in an oil bath (~190 °C) for 1.5–2 h. Pure *exo*-adduct was obtained after three successive recrystallizations from benzene (11.06 g, 22%), mp 140–142 °C; IR (KBr) ν_{max} 1860, 1777, 1218, 1086 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.53 (2H, m, 7-H_2), 2.99 (2H, m, 1-H and 4-H), 3.64 (2H, m, 2-H and 3-H), 6.32 (2H, m, 5-H and 6-H) ppm.

4.1.3. *exo*-Bicyclo[2.2.1]heptane-2,3-dicarboxyclic anhydride (Ligand C). *exo*-Bicyclo[2.2.1]hept-5-ene-2,3-dicarboxylic anhydride (4.75 g, 0.029 mol) was dissolved in ethyl acetate (50 mL) and Pd–C (10%, 0.47 g) added. Hydrogen gas was introduced into the reaction flask at atmospheric pressure via a balloon, and the mixture stirred at room temperature for ~5 h. The mixture was filtered and ethyl acetate was evaporated in vacuo. The resultant white solid was recrystallized from ethanol to yield Ligand **C** (2.76 g, 57%), mp 80–82 °C; IR (KBr) ν_{max} 1862, 1788, 1227, 1089, 910 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.28–1.39 (4H, m, 5-H_2 and 6-H_2), 1.69 (2H, m, 7-H_2), 2.82–2.89 (4H, m, 1-H to 4-H) ppm; ^{13}C NMR ($CDCl_3$) δ 27.8 (C-5 and C-6), 34.8 (C-7), 41.4 (C-1 and C-4), 49.5 (C-2 and C-3), 173.4 (CO) ppm; HRMS (CI): calcd for $C_9H_{10}O_3$ [MH^+] 167.0703; found: 167.07027.

4.1.4. Preparation of compound A. K_2PtCl_4 (0.84 g, 1.93 mmol) was dissolved in water (15 mL), heated to 80 °C and KI (1.92 g, 11.6 mmol) was added into the solution and mixture was allowed to react in the dark for 15 min. The reaction mixture was then cooled to 40 °C and NH_3 (30% aq solution) added dropwise with stirring and pH maintained at 8, until a dark yellow precipitate formed. The mixture was further stirred at 40 °C for 2 h, filtered and precipitate washed with water (5 mL), ethanol (5 mL) and diethyl ether (5 mL) to produce $(NH_3)_2PtI_2$ (0.88 g, 95%). $(NH_3)_2PtI_2$ (0.48 g, 1 mmol) was suspended in water (15 mL) and $AgNO_3$ (0.68 g, 4 mmol, in 5 mL water) was added and the mixture stirred in the dark overnight, filtered, and solid AgI removed. Ligand **C** (0.166 g, 1 mmol) and NaOH (0.08 g, 2 mmol) were added into the filtrate and mixture stirred for 8 h at room temperature. A white solid was formed, filtered and washed with water (5 mL), ethanol (5 mL), and diethyl ether (5 mL) to give Compound **A** (0.2 g, 45%), which completely decomposed at 270 °C; IR (KBr) ν_{max} 3435, 3271, 1617, 1570, 1384 cm^{-1}. Elemental analysis (CHN): calcd for $C_9H_{16}N_2O_4Pt{\cdot}H_2O$ (%): C, 25.17; H, 4.20; N, 6.53; found: C, 25.00; H, 4.21; N, 6.36.

4.1.5. Preparation of compound B. Compound **B** was similarly synthesized from K_2PtCl_4 (1 g, 2.41 mmol) and *trans*-(±)-1,2-diaminocyclohexane (DACH) (0.275 g, 2.41 mmol). Compound **B** (0.36 g, 73%), which completely decomposed at 280 °C; IR (KBr) ν_{max} 3412, 3223, 2943, 1571, 1384 cm^{-1}. Elemental analysis (CHN): calcd for $C_{15}H_{24}N_2O_4Pt{\cdot}2H_2O$ (%): C, 34.16; H, 5.31; N, 5.31; found: C, 34.48; H, 5.14; N, 5.24.

4.2. Cell lines

Mouse leukemia L1210 and human hepatoma SK-Hep-1 cell lines were obtained from the American Type Culture Collection. L1210 cells were grown in suspension culture in RPMI 1640 medium supplemented with fetal bovine serum (10%), penicillin (100 U/mL) and streptomycin (100 μg/mL). SK-Hep-1 cells were grown as adherent culture in DMEM medium supplemented with fetal bovine serum (10%), penicillin (100 U/mL) and streptomycin (100 μg/mL). The cells were grown in a CO_2 incubator (5% CO_2/95% air) at 37 °C.

4.3. Induction of cisplatin resistance

Cisplatin-resistant L1210 leukemia cell line (L1210/CDDP) was developed over a time period exceeding 18 months. For the induction of cisplatin resistance in the human hepatoma cell line SK-Hep-1, the cells were treated weekly with 10 μM cisplatin for 1 h.[30] After 10 months, stable acquired-resistant variants (SK-Hep-1/$CDDP_{10}$) were established and subsequently cloned. At the time of experimentation, L1210/CDDP cells were 30-fold resistant to cisplatin and 12-fold resistant to carboplatin; and SK-Hep-1/$CDDP_{10}$ cells were 5-fold resistant to cisplatin and 3.5-fold resistant to carboplatin. All experiments involving cisplatin-resistant cell lines were performed after incubating cells in a drug-free medium for 2 weeks.

4.4. Inhibition of protein phosphatase activity in tumor cell homogenates

Inhibition of protein phosphatases (PP1 and PP2A) in whole cell homogenates of sensitive and cisplatin-resistant L1210 leukemia and SK-Hep-1 hepatoma cells were determined in triplicate.[13] In brief, approximately 2×10^7 wild-type L1210/0 and cisplatin-resistant L1210/CDDP cells were collected by centrifugation (1000 g, 5 min); and 4×10^6 wild-type SK-Hep-1/0 and

cisplatin-resistant SK-Hep-1/CDDP$_{10}$ cells were collected by scratching from culture dishes. The cell pellets were homogenized, washed thoroughly in ice-cold Ringer solution to remove free phosphate, and whole cell homogenates were then further diluted with ice-cold Ringer solution.[31] This was followed by the addition of 1 mM EDTA to inhibit the PP2B/PP2C.

The TCM–Pt compounds, DMC, Ligand **C**, compounds **A** and **B**, cisplatin or carboplatin was added and the reaction was started with the addition of the pseudo-substrate phosphopeptide KR (phospho-T)IRR (K-R-pT-I-R-R) (Upstate Biotechnology, Inc) at a concentration of 200 μM in a final volume of 25 μL. All enzyme assays were performed in 96-well plates at room temperature. After a 30-min incubation the dephosphorylation reaction was stopped by the addition of malachite green solution (100 μL), and after 10 min absorbance was measured at 650 nm using a microtiter plate reader.[14] Free phosphate was quantified by comparison to a Pi-malachite green standard curve. Protein phosphatase activity in the whole cell homogenates with or without inhibition by the TCM–Pt compounds **1**–**5** and DMC was expressed as nanomoles phosphate (Pi) released per milligram protein per minute. The total amount of protein phosphatases in the cell homogenates was determined by using okadaic acid, a specific PP2A inhibitor. The percentage inhibition of protein phosphatases by the novel compounds was calculated based on the total amount of protein phosphatases as determined by okadaic acid inhibition. The concentration of drug resulting in 50% inhibition of phosphatase activity (IC_{50}) was determined using the software Graphpad Prism 3.0 by fitting the graph into sigmoidal dose–response curves.

4.5. In vitro antiproliferative assay

The growth inhibitory activity of the platinum compounds together with DMC and ligand **C** was assayed using the colorimetric MTT assay.[26] Briefly, cells were seeded in 96-well microtitre plates (100 μL) and allowed to equilibrate in quadruplicate wells and exposed to drugs for 72 h. After which, MTT (5 mg/mL, 20 μL) in phosphate buffered saline was added and the cells were incubated for 4 h at 37 °C. MTT/medium was then removed and the formazan product was dissolved in dimethylsulfoxide (DMSO, 150 μL) and absorbance was measured at 570 nm using a microtitre plate reader. The concentration of drug resulting in 50% growth inhibition (IC_{50}) was determined using Graphpad Prism 3.0 by fitting the graph into sigmoidal dose–response curve.

4.6. Repair of DNA-platinum lesions

Briefly, sensitive parental and cisplatin-resistant L1210 cells treated with 100 μM platinum compounds were washed three times with ice-cold PBS and frozen at −80 °C (zero time control) or re-incubated in drug-free medium for 2, 6, 12, and 24 h to allow for repair. After the repair incubations, the cells were washed twice more with cold sterile PBS and again frozen at −80 °C until DNA was isolated by using DNAzol® reagent. The total DNA bound platinum was estimated by atomic absorption spectroscopy (wavelength: 265.9 nm; apparatus: Perkin–Elmer Analyst 100 atomic absorption spectrometer with graphite cell atomizer HGA800), with a method similar to that described by Di Blasi et al.[32] Repair was calculated by comparing the platinum content for the repair samples with that in the zero time control. The removal of platinum adducts from DNA following washing out of the drug and re-incubation for 2, 6, 12, and 24 h was used as a functional assay of overall cellular nucleotide excision repair (NER) activity. Platination measurements were made in quadruplicate where the standard error was consistently less than 10%. All experiments were repeated three times. To assess the role of demethylcantharidin in the TCM–Pt compounds, DNA platination and repair assays were repeated with combination of cisplatin (100 μM) and demethylcantharidin (6 μM) added concomitantly.

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